

Available online at [www.sciencedirect.com](http://www.sciencedirect.com)

SCIENCE @ DIRECT®

Biochimica et Biophysica Acta 1661 (2004) 204–211



# Properties of the basal calcium influx in human red blood cells

Roman Hudec<sup>a,1</sup>, Boris Lakatoš<sup>a,1</sup>, Karin Kaiserová<sup>a</sup>, Jozef Orlický<sup>b</sup>, L'udovít Varečka<sup>a,\*</sup>

<sup>a</sup>Department of Biochemistry and Microbiology, Slovak University of Technology, Radlinského 9, Bratislava 812 37, Slovak Republic

<sup>b</sup>Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, 833 34 Bratislava, Slovak Republic

Received 22 July 2003; received in revised form 5 January 2004; accepted 27 January 2004

## Abstract

The basal  $^{45}\text{Ca}^{2+}$  influx in human red blood cells (RBC) into intact RBC was measured.  $^{45}\text{Ca}^{2+}$  was equilibrated with cells with  $t_{1/2} = 15\text{--}20\text{ s}$  and the influx reached the steady state value in about  $90\text{--}100\text{ s}$  and the steady state level was  $1.5 \pm 0.2\text{ }\mu\text{mol/l}_{\text{packed cells}}$  ( $n=6$ ) at  $37\text{ }^{\circ}\text{C}$ . The average value of the  $\text{Ca}^{2+}$  influx rate was  $43.2 \pm 8.9\text{ }\mu\text{mol/l}_{\text{packed cells}}\text{ hour}$ . The rate of the basal influx was pH-dependent with a pH optimum at pH 7.0 and on the temperature with the temperature optimum at  $25\text{ }^{\circ}\text{C}$ . The basal  $\text{Ca}^{2+}$  influx was saturable with  $\text{Ca}^{2+}$  up to  $5\text{ mmol/l}$  but at higher extracellular  $\text{Ca}^{2+}$  concentrations caused further increase of basal  $\text{Ca}^{2+}$  influx. The  $^{45}\text{Ca}^{2+}$  influx was stimulated by addition of submicromolar concentrations of phorbol esters (phorbol 12-myristate-13-acetate (PMA) and phorbol-12,13-dibutyrate (PDBu)) and forskolin. Uncoupler (3,3',4',5-tetrachloro-salicylanilide (TCS)  $10^{-6}\text{--}10^{-5}\text{ mol/l}$ ) inhibited in part the  $\text{Ca}^{2+}$  influx. The results show that the basal  $\text{Ca}^{2+}$  influx is mediated by a carrier and is under control of intracellular regulatory circuits. The effect of uncoupler shows that the  $\text{Ca}^{2+}$  influx is in part driven by the proton-motive force and indicates that the influx and efflux of  $\text{Ca}^{2+}$  are coupled via the RBC  $\text{H}^{+}$  homeostasis.

© 2004 Elsevier B.V. All rights reserved.

**Keywords:** Red blood cell; Basal  $\text{Ca}^{2+}$  influx; Uncoupler

## 1. Introduction

$\text{Ca}^{2+}$  ions enter the cytoplasm after the membrane receptors and/or channels are activated by a signalling event [1–3]. On the other hand,  $\text{Ca}^{2+}$  also enters the cytoplasm without stimulation by external stimuli (including the membrane potential changes) and this could be revealed experimentally, e.g. by labelling of cells with  $^{45}\text{Ca}^{2+}$ . This process is denoted as the basal  $\text{Ca}^{2+}$  influx. In contrast to ligand- or potential-induced changes in  $\text{Ca}^{2+}$  fluxes, the basal  $\text{Ca}^{2+}$  is much less referred to in the literature, usually mentioned as control. The rates of basal  $\text{Ca}^{2+}$  influx vary in magnitude (expressed in  $\text{mol/mg protein}$ ). The highest

influx was observed in synaptosomes [4], but was lower [5,6] in blood platelets. The magnitudes and properties of the basal  $\text{Ca}^{2+}$  influx may represent a characteristic feature (parameter) of the cell and probably is under genetic control. However, the systematic and exhausting data, which could elucidate the processes underlying the basal  $\text{Ca}^{2+}$  influx, are still missing.

Human red blood cells (RBC) are a prototype of cells, which exhibit the basal  $\text{Ca}^{2+}$  transport only, as their membranes do not possess receptor-mediated functions and they apparently do not contain cytoplasmic organelles. There are also numerous reports describing the character of the basal  $\text{Ca}^{2+}$  transport. Lew et al. [7], McNamara and Wiley [8] and also Pokudin and Orlov [9] have measured the rates of the  $^{45}\text{Ca}^{2+}$  influx in presence of calcium chelator in cells. These papers showed the saturability of the transport and its insensitivity to the monovalent cation mediums composition. This approach although enables comfortable measurement, but probably does not yield a true value of the rates due to the (additional) buffering of the cytoplasm, which may distort the original concentration conditions and increases the total  $\text{Ca}^{2+}$  accumulation in the cytoplasm [10].

**Abbreviations:** DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; diSC<sub>3</sub>-(5), 3,3'-dipropylthiodicarbocyanine iodide; DMSO, dimethyl sulfide; EDTA, ethylene diamine-*N,N'*-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; PDBu, phorbol-12,13-dibutyrate; PMA, phorbol-12-myristate-13-acetate; TCS, 3,3',4',5-tetrachloro-salicylanilide

\* Corresponding author. Tel.: +42-1-2-59325-514; fax: +42-1-2-52493-198.

E-mail address: [ludovit.varecka@stuba.sk](mailto:ludovit.varecka@stuba.sk) (L. Varečka).

<sup>1</sup> Both authors contributed equally to this work.

Desai et al. [11] reported the description of the  $\text{Ca}^{2+}$  influx observed after eliminating the active  $\text{Ca}^{2+}$  extrusion and found that it matches the known rate of the  $\text{Ca}^{2+}$  efflux. Engelmann et al. [12] measured the  $\text{Ca}^{2+}$  influx without entrapping the  $\text{Ca}^{2+}$  chelator and showed that the kinetics of the basal  $\text{Ca}^{2+}$  influx is faster than that in  $\text{Ca}^{2+}$  chelator-loaded cells and could be stimulated by external ligand, epidermal growth factor. Varečka et al. [13] found that the basal  $^{45}\text{Ca}^{2+}$  measured without entrapped  $\text{Ca}^{2+}$  chelator is not sensitive to the changes in external  $\text{Na}^+$  and  $\text{K}^+$  concentrations. Recently, Andrews et al. [14] using the fura-2 fluorescence measurements published a more detailed description of the basal  $\text{Ca}^{2+}$  influx in human RBCs and shown that it is stimulated by phorbol-12-myristate-13-acetate (PMA), an activator of protein kinase C (PKC). Soldati et al. [15] used the same approach and suggested that the  $\text{Ca}^{2+}$  influx is mediated by membrane potential-operated channel, and, in another paper [16], studied the mechanism of the parathyroid hormone on the calcium influx. However, the complete transport characteristics of the basal  $\text{Ca}^{2+}$  influx are still missing.

The problem intimately connected with the existence of the basal  $\text{Ca}^{2+}$  influx follows from the charge-compensating properties of the  $\text{Ca}^{2+}$ -ATPase, which according to several

studies [17–19], catalyses the ATP-dependent electroneutral  $\text{Ca}^{2+}/2\text{H}^+$ , or partially compensated  $\text{Ca}^{2+}/\text{H}^+$  exchange [20]. This implies that there is net influx of one to two protons after each cycle of basal  $\text{Ca}^{2+}$  influx. Such an influx might, if not compensated, dramatically change cytoplasmic pH in the steady state. Another implication of these properties of the  $\text{Ca}^{2+}$ -ATPase is that there is a net influx of charge during each cycle of the cyclic  $\text{Ca}^{2+}$  flux. This aspect has been also analysed, in addition to the basic properties of the  $\text{Ca}^{2+}$  influx, in this paper.

## 2. Materials and methods

### 2.1. Red blood cell suspension

Blood from healthy volunteers of both sexes was withdrawn by venipuncture into acid-citrate-dextrose anti-coagulant in the local blood transfusion station and was used at a second day after blood withdrawal being stored at 0–4 °C. RBC were isolated after centrifugation of the blood (10 min at 600 × g), an aspiration of the supernatant with the buffy coat and three-fold washing and, finally, suspending into a medium containing (in mmol/

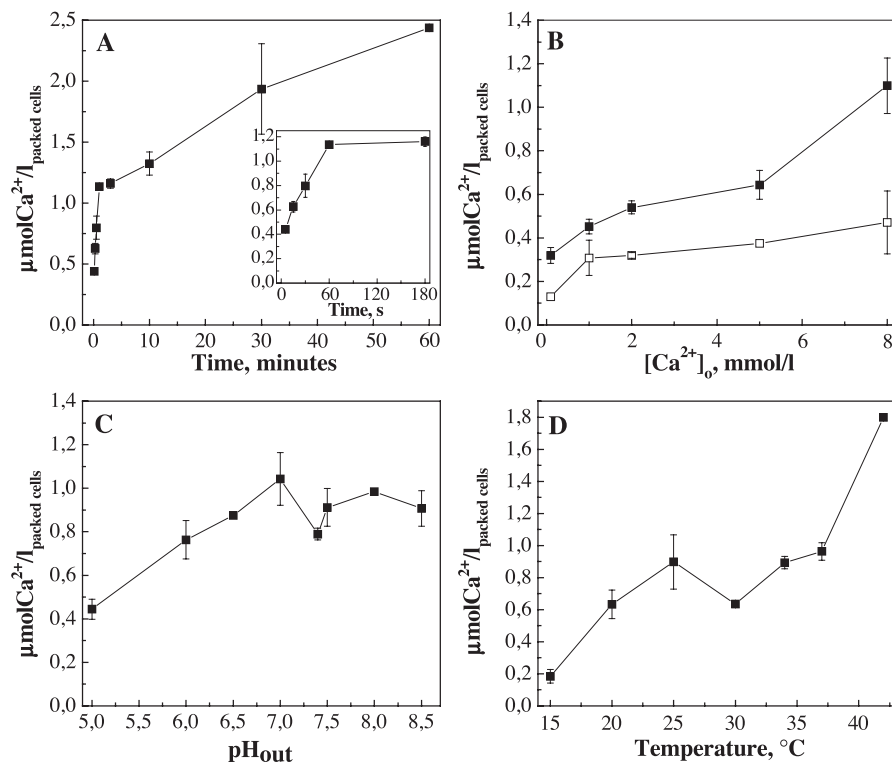


Fig. 1. Basic characteristics of  $^{45}\text{Ca}^{2+}$  influx into human red blood cells. (A) Kinetics of  $^{45}\text{Ca}^{2+}$  influx was measured by addition of 2.5 mmol/l  $\text{Ca}^{2+}$  at 37 °C. Inset shows data of initial phase of the time dependence of basal influx re-plotted in expanded time scale. (B) Concentration dependence of  $\text{Ca}^{2+}$  influx at 37 °C at zero time (□) and after 3 min (■). (C) Dependence of  $^{45}\text{Ca}^{2+}$  influx at 37 °C with 2.5 mmol/l  $\text{Ca}^{2+}$  on external pH. (D) Temperature dependence of  $^{45}\text{Ca}^{2+}$  influx was measured 10 min after addition of radionuclide. Concentration of  $\text{Ca}^{2+}$  was 2.5 mmol/l. In panels C and D, the values are corrected for measurement at time zero. All plots are representative from the independent experiments with 30% RBC suspensions. ( $n_A=6$ ,  $n_B=3$ ,  $n_C=2$ ,  $n_D=4$ ). Points are mean  $\pm$  S.E. of duplicates.

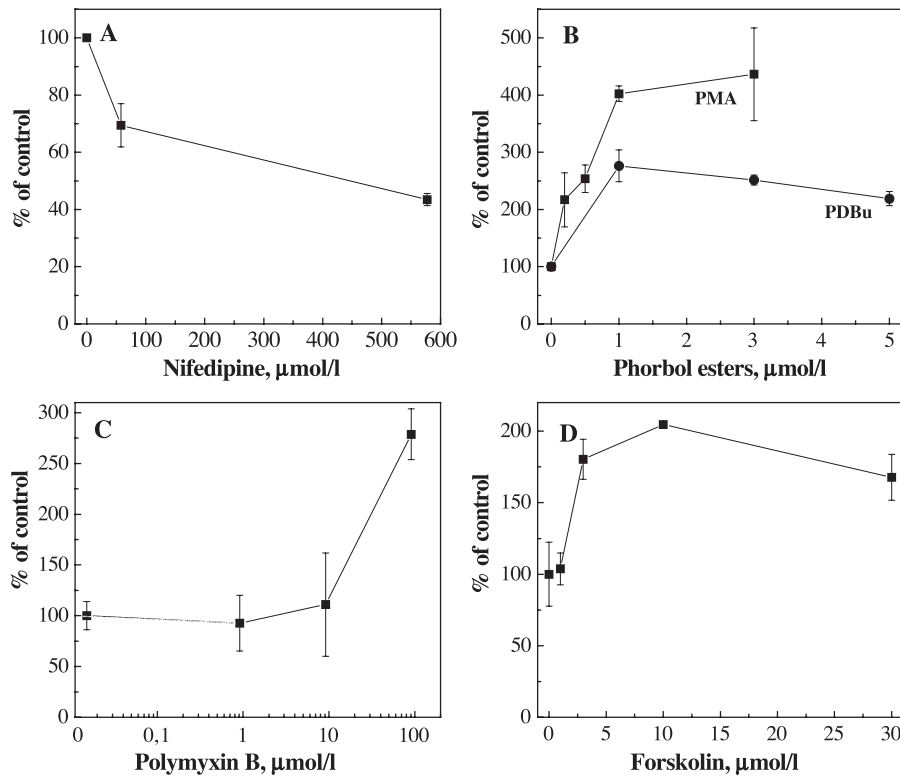


Fig. 2. Effect of compounds modulating signalling pathways on the basal  $^{45}\text{Ca}^{2+}$  influx into human red blood cells. (A) Effect of nifedipine on basal  $\text{Ca}^{2+}$  influx. (B) Effect of phorbol esters (PMA (■) and PDBu (●)) on basal  $\text{Ca}^{2+}$  influx. (C) Effect of polymyxin B on basal  $\text{Ca}^{2+}$  influx. (D) Effect of forskolin on basal  $\text{Ca}^{2+}$  influx. All experiments were measured under identical conditions: radionuclide final concentration 2.5 mmol/l, 30% cell suspension, 37 °C, concentrations of additions are indicated in the figures. Plots are representative from independent experiments ( $n > 2$ ). Points are mean  $\pm$  S.E. of duplicates and corrected for values obtained at time zero.

l): 20 Tris–Cl (pH 7.4); 130 NaCl; 5 KCl; 1.2  $\text{NaH}_2\text{PO}_4$ , 1  $\text{MgCl}_2$  and 10 glucose (further referred to as the suspension medium), to the final haematocrit of 30%, and immediately used for experiment.

## 2.2. Measurement of the basal $^{45}\text{Ca}^{2+}$ influx

The procedure described by Pokudin and Orlov [9] was modified as follows: red cells (0.5 ml, 30% haematocrit)

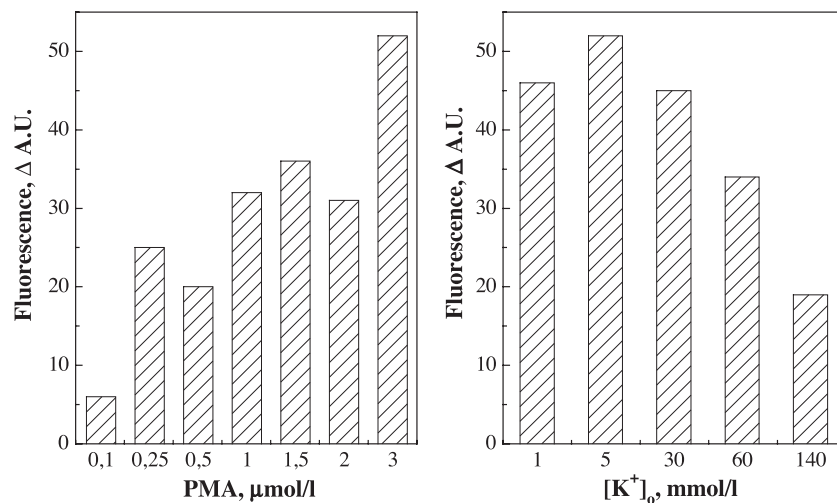


Fig. 3. Effects of PMA on fluorescence of diSC<sub>3</sub>-(5) in the suspension of human erythrocytes. Changes in the fluorescence  $F_{651/675}$  were measured using 3.6  $\mu\text{mol/l}$  diSC<sub>3</sub>-(5) and 20% suspension of red cells in the suspension medium. Left panel: fluorescence change depended on the PMA concentration in presence of 5 mmol/l  $\text{K}^+$  in medium. Right panel: fluorescence change depended on concentration of extracellular potassium after addition of the maximal efficient concentration of PMA (3  $\mu\text{mol/l}$ ). In both panels, changes in  $[\text{K}^+]_{\text{ex}}$  were compensated with  $\text{Na}^+$  to isotonicity.

were pre-incubated at 37 °C,  $^{45}\text{Ca}^{2+}$  (2.5 mmol/l; spec. act. approx. 4000–8000 cpm/nmol) was added, and incubation was continued at 37 °C. At times indicated (or after 20 min when not indicated specifically) 0.5 ml aliquots of the suspension were withdrawn and added to 5 ml of medium containing (in mmol/l): 20 Tris–Cl (pH 7.4); 65 NaCl; 70 KCl; 5 EGTA and 10 glucose cooled in an ice–water mixture, and immediately spun down on a microcentrifuge at 2–4 °C (the experiments were carried out in a cold room). Two more washing steps followed, and the radioac-

tivity of pellets was determined after precipitation of cells with 0.5 ml of 10% trichloroacetic acid containing 10 mmol/l  $\text{LaCl}_3$  and centrifugation of precipitates (5 min,  $12,000 \times g$ ). The substances to be tested were added 5 min prior to the addition of radionuclide.

### 2.3. The measurement of the membrane potential changes

These changes were measured by means of the fluorescence probe 3,3'-dipropylthiodicarbocyanine iodide ( $\text{diSC}_3$ -

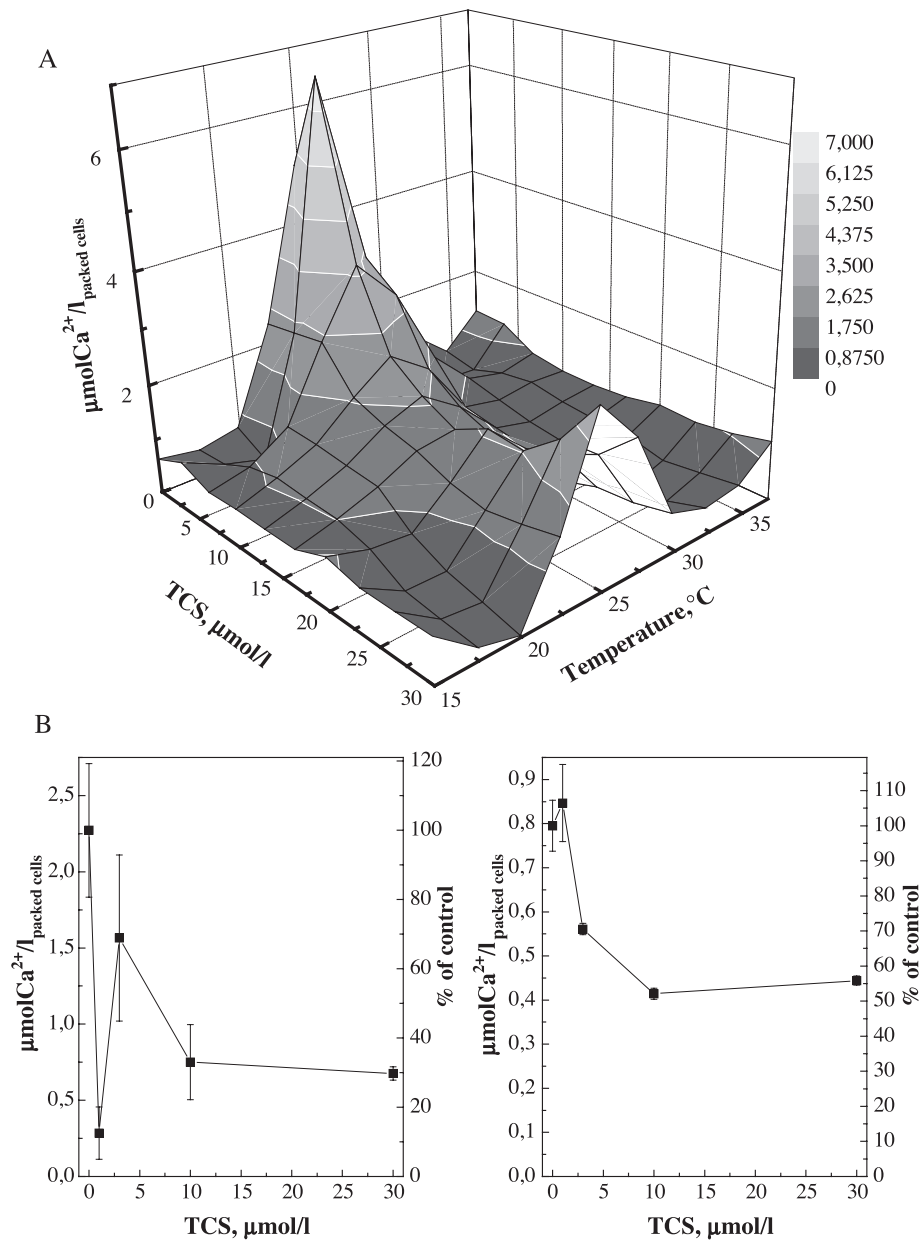


Fig. 4. The effect of TCS on basal  $^{45}\text{Ca}^{2+}$  influx into human red blood cells. (A) Three-dimensional plot describes the effect of TCS in the concentration range 0–30  $\mu\text{mol/l}$  on the basal  $^{45}\text{Ca}^{2+}$  influx at temperatures between 15 and 37 °C (1% methanol was present in control samples treated in parallel). Other conditions as described in Materials and methods. Gray scale on the right site is in  $\mu\text{mol Ca}^{2+}/\text{l packed cells}$ . Area was corrected for measurements at time zero. The pilot experiment. (B) The dependence of the basal  $^{45}\text{Ca}^{2+}$  influx on the concentration of TCS at 30 °C (left panel) and 37 °C (right panel). Experimental conditions as above. Representative from independent experiments ( $n=6$ ). Data in panels A and B are from independent experiments. Points are mean  $\pm$  S.E. of duplicates and corrected for measurement at time zero.

(5)) as described by Sims et al. [21] using 651 and 675 nm as excitation and emission wavelengths, respectively. The measurements were performed in the continuously stirred thermostatted cuvette in a Carl Zeiss (Opton) PMQ fluorometer.

#### 2.4. Materials

$^{45}\text{CaCl}_2$  from ICN, USA; valinomycin from Calbiochem, Luzern, Switzerland; forskolin, phorbol-12-myristate-13-acetate (PMA), phorbol-12,13-dibutyrate (PDBu) and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) from Sigma; Tris base and polymyxin B from Serva; 3,3',4',5-tetrachloro-salicylanilide (TCS) from Eastman-Kodak; diSC<sub>3</sub>-(5) from Fluka, Buchs, Switzerland and dimethyl sulfoxide (DMSO) from Applichem, Darmstadt, Germany. Other chemicals used (all of analytical grade) were purchased from Lachema, Brno, Czech Republic.

### 3. Results

The addition of  $^{45}\text{Ca}^{2+}$  into the RBC suspension kept at 37 °C led to its rapid equilibration with cells, which was completed after about 90–100 s (Fig. 1A). A prolonged incubation induced the gradual and slow increase of cell radioactivity. After 1 h of incubation the cell  $\text{Ca}^{2+}$  content attained the value up to 2.5  $\mu\text{mol/l}_{\text{packed cells}}$ . The uptake was saturable by  $\text{Ca}^{2+}$  concentrations smaller than 5 mmol/l (Fig. 1B) but the basal  $^{45}\text{Ca}^{2+}$  influx was further stimulated upon exceeding this  $\text{Ca}^{2+}$  concentration (Fig. 1B). The basal  $^{45}\text{Ca}^{2+}$  influx was maximal at pH 7.0 and was moderately inhibited by lowering (to pH 5) or increasing (to pH 8.5) the pH of the medium (Fig. 1C). The basal  $\text{Ca}^{2+}$  influx was stimulated by the increase of temperature (Fig. 1D) with the optimum at 25 °C. Over this temperature the  $\text{Ca}^{2+}$  influx decreased, although at 42 °C, another phase of stimulation appeared (Fig. 1D). In some experiments, only an ascending phase of the  $\text{Ca}^{2+}$  influx was observed (not shown). From the measurements of the  $\text{Ca}^{2+}$  influx temperature dependencies ( $n=4$ ) was calculated the activation energy of 62 kJ/mol a temperature quotient  $Q_{10}$  of 1.3 by an Arrhenius plot of the ascending part of the curves.

The basal  $^{45}\text{Ca}^{2+}$  influx was inhibited by nifedipine (up to 0.58 mmol/l) (Fig. 2A). The degree of inhibition (max. 55%) was substantially lower than that observed in the vanadate-induced  $^{45}\text{Ca}^{2+}$  influx [22]. Furthermore, the effect of other compounds with possible effect on calcium influx was studied. Phorbol derivatives, PMA and PDBu exerted a stimulatory effect in the submicromolar concentration range, PMA with the half-maximal stimulation at 0.5  $\mu\text{mol/l}$  (Fig. 2B). The extent of the stimulation was from 2.3 to 5 times in various experiments. Polymyxin B, PKC inhibitor, activated the basal  $^{45}\text{Ca}^{2+}$  influx at concentrations higher than 10  $\mu\text{mol/l}$  (Fig. 2C). Forskolin, activator of adenylate cyclase, also exerted a stimulatory effect on the basal  $^{45}\text{Ca}^{2+}$  influx (Fig. 2D) with the half-maximal stim-

ulation at about 2  $\mu\text{mol/l}$ . Several other compounds were tested for their effect on the basal  $^{45}\text{Ca}^{2+}$  influx. Secalonic acid D, a teratogen and PKC inhibitor, rugulosin and scopathricin (mycotoxins) exerted only a minor stimulatory effect (about 15%) in the concentration range 0–50  $\mu\text{mol/l}$ . Gliotoxin (apoptosis inducer in liver cells) [23] had a minor inhibitory effect (about 10%) on the basal  $\text{Ca}^{2+}$  influx (not shown).

PMA, if added to the suspension of RBCs equilibrated with the membrane potential fluorescence probe diSC<sub>3</sub>-(5), induced a concentration-dependent increase of the fluorescence. This increase was found to be dependent on the concentration of  $\text{K}^+$  with a biphasic response (Fig. 3) being maximal at 5 mmol/l  $\text{K}^+$  and inhibited at higher  $\text{K}^+$  concentrations (completed with  $\text{Na}^+$  to isotonicity) suggesting that the effect of PMA could be ascribed to the depolarisation the membrane (Fig. 3).

The fact that  $\text{Ca}^{2+}$ -ATPase catalyses the ATP-driven  $\text{Ca}^{2+}/1-2\text{H}^+$  exchange suggests that the basal  $^{45}\text{Ca}^{2+}$  influx should be influenced by the permeability to  $\text{H}^+$  of the human RBC membrane. Therefore, the next experiments were carried out to test the effect of uncoupler on the basal  $^{45}\text{Ca}^{2+}$  influx. The last was sensitive to uncoupler, TCS, which inhibited the basal  $^{45}\text{Ca}^{2+}$  influx up to 60% in various experiments (Fig. 4A). The maximal degree of inhibition was obtained at 10  $\mu\text{mol/l}$  TCS and apparently occurred at various temperatures. At lower concentrations (e.g. 2–5  $\mu\text{mol/l}$ ) irregular phenomena appeared, which were different from one experiment to another and which were characteristic of the scattering of experimental points of the basal  $\text{Ca}^{2+}$  influx (Fig. 4B) indicating that the

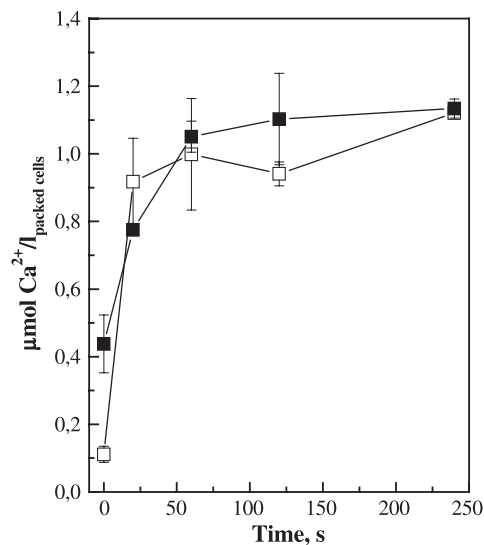


Fig. 5. The effect of DIDS on basal  $^{45}\text{Ca}^{2+}$  influx into human red blood cells. Kinetics of the basal  $^{45}\text{Ca}^{2+}$  influx was measured as described in Materials and methods in the presence (■) or absence (control, 0.1% DMSO (□)) of 25  $\mu\text{mol/l}$  DIDS in 30% human RBC suspension at 37 °C. Plot is representative from three independent experiments. Points are mean  $\pm$  S.E. of duplicates.



presence of uncoupler induces an instability in the extent of the  $^{45}\text{Ca}^{2+}$  influx. The degree of inhibition varied among experiments between 40% and 80% ( $n = 10$ ). In order to find out whether the anion channel, the site of  $\text{Cl}^-/\text{OH}^-$  exchange [24], plays a role in the basal  $\text{Ca}^{2+}$  influx, the effect of anion channel inhibitor DIDS was tested. DIDS did not cause a significant effect on the basal  $^{45}\text{Ca}^{2+}$  influx at the concentration of 25  $\mu\text{mol/l}$  (Fig. 5). In a pilot experiment, valinomycin (1  $\mu\text{g/ml}$ ), stimulated the basal  $\text{Ca}^{2+}$  influx by 15%, however, its stimulatory effect was observed also in  $\text{K}^+$ -containing medium (not shown).

#### 4. Discussion

The results presented above demonstrate that the basal  $\text{Ca}^{2+}$  influx occurs in the membrane of human RBC, which has all characteristics of the carrier-mediated transport. As the  $\text{Ca}^{2+}$  efflux is mediated solely by the  $\text{Ca}^{2+}$ -ATPase (there is no evidence for the presence of a  $\text{Na}^+/\text{Ca}^{2+}$  antiporter in human red cells), then it is possible to ascribe the  $\text{Ca}^{2+}$  influx to a cyclic flow of  $\text{Ca}^{2+}$ , which occurs without added stimuli and which consists of these transport systems. This statement could be supported not only by the transport characteristics shown in Fig. 1 but also by its magnitude which was rather high and varied between 20.8 and 47.6  $\mu\text{mol/l}_{\text{packed cells h}}$  at 37 °C and falls into the range observed under similar conditions by other authors. From the paper of Engelmann et al. [12] the value between 30 and 40  $\mu\text{mol/l}_{\text{packed cells h}}$  could be derived from data shown in Fig. 2B at 37 °C. On the other hand, other papers where the  $^{45}\text{Ca}^{2+}$  influx into RBC containing entrapped  $\text{Ca}^{2+}$  chelators was measured presented different but essentially similar results: 2–20, 14–20, 59  $\mu\text{mol/l}_{\text{packed cells h}}$  [7–9], respectively, less than 50  $\mu\text{mol/l}_{\text{packed cells h}}$  [25]. Here we do not discuss the values of the  $\text{Ca}^{2+}$  influx in ATP-depleted and vanadate-treated cells, where the net  $\text{Ca}^{2+}$  accumulation occurs and the qualitative changes in properties of the  $\text{Ca}^{2+}$  transport were observed [22,26,27]. Under conditions used in this work, no inhibition of  $\text{Ca}^{2+}$ -ATPase is expected. Concentration of ATP was found to be 0.6 mmol ATP/ $\text{l}_{\text{packed cells}}$  (unpublished results). In this case, the net  $\text{Ca}^{2+}$  accumulation does not take place (Fig. 1A) and only the turnover of calcium cyclic flow is measured.

The kinetics of the  $^{45}\text{Ca}^{2+}$  influx show the clear biphasicity, which was not observed before (Fig. 1A). The rapid phase has similar time-course as that observed by Engelmann et al. [12]. The second phase may indicate that the longer pre-incubation of RBC with  $^{45}\text{Ca}^{2+}$  in artificial medium leads to the slow accumulation, and possibly the precipitation of  $^{45}\text{Ca}^{2+}$  in the cytoplasm. The temperature optimum of the basal  $^{45}\text{Ca}^{2+}$  influx was similar to that induced by vanadate [28] and could be explained similarly, i.e. by the differences in the temperature sensitivities of both transports systems participating in the cyclic  $\text{Ca}^{2+}$  flow.

Unlike our [13,22] and other [26] findings, in presented experiments we observed an inhibition of the basal  $^{45}\text{Ca}^{2+}$  by nifedipine (Fig. 2A) up to 55% as compared with about 10–20% inhibition in the quoted papers. This difference is probably to explain by the fact that in this work, we used cells which were stored 1 day since the blood withdrawal.

The question of the physiological role of the basal  $\text{Ca}^{2+}$  influx remains strictly speaking essentially unanswered. However, some experimental results provide useful hints in this respect. First, the effects of phorbol esters [14], Fig. 2B in this work, and the effects of polymyxin B and forskolin (Fig. 2C and D) indicate that changes in intracellular variables of signalling pathways mediated by PKC, or adenylate cyclase, respectively, can modify the rate of the basal influx. These changes occur without involvement of a specific receptor, therefore, these signalling pathways may represent remnants of those present in the preceding developmental stages of erythropoiesis, or they reflect interventions from a functional intracellular signalling pathway(s). These explanations have to be tested in the future.

The stimulatory effect of PMA on the basal could be explained by a direct stimulation by phosphorylation of the putative  $\text{Ca}^{2+}$  carrier or a hypothetical regulatory protein(s). It seems to be a feasible explanation as we found several proteins phosphorylated by PMA at conditions similar to those used in this study (unpublished results). However, it was found that PMA caused the depolarisation of the membrane (Fig. 3, left panel). Thus, alternative explanation is possible that the depolarisation-induced  $\text{Ca}^{2+}$  influx is activated with PMA, in a  $\text{Na}^+$ -dependent manner (Fig. 3, right panel). Such an explanation is in agreement with data of Soldati et al. [15], but there are no data which could discriminate between these possibilities.

The observation of Niggli et al. [17], that the  $\text{Ca}^{2+}$ -ATPase catalyses the electrically silent  $\text{Ca}^{2+}/2\text{H}^+$  exchange, reveals an important problem intimately connected with the existence of RBC basal  $\text{Ca}^{2+}$  transport. It could be inferred that the cyclic flow of  $\text{Ca}^{2+}$  (of the magnitude indicated above) should lower the cytoplasmic pH by several orders of magnitude within 1 h (down to pH 3.3). Such a change apparently does not happen in RBC under normal conditions. The inhibitory effect of the uncoupler on the basal  $\text{Ca}^{2+}$  influx (Fig. 4) indicates that the  $\text{H}^+$  electrochemical potential contributes to the driving force of the basal  $\text{Ca}^{2+}$  influx. This effect of uncoupler cannot be expected if the basal  $\text{Ca}^{2+}$  influx pathway is mediated by an uniport (i.e. channel-like) transport system as the inflow of  $\text{H}^+$  (due to the  $\text{Ca}^{2+}$ -ATPase activity) should be compensated by a mechanism which is located outside of both transport proteins involved in the basal  $\text{Ca}^{2+}$  cyclic flow excluding, however, the anion channel (Fig. 5).

The most plausible interpretation of the effect of uncoupler is that it releases the constraint of  $\text{Ca}^{2+}$ -ATPase due to the influx of  $\text{H}^+$ , and accelerates efflux of  $\text{Ca}^{2+}$ . This should mask a part of the  $\text{Ca}^{2+}$  influx and decrease the radioactivity in cells. This explanation, however, also has some weak-

nesses. First, the capacity of the  $\text{Ca}^{2+}$ -ATPase exceeds the capacity of the  $\text{Ca}^{2+}$  influx by order of magnitude (see Ref. [25] for review). Second, this interpretation does not explain the net flux of two charges (carried by entering  $\text{Ca}^{2+}$ ) per transport cycle.

There are two other ways to explain the effect of uncoupler; though it must be said that none of them is fully supported by experimental data. The first explanation is based on the inhibition of the vanadate-induced  $^{45}\text{Ca}^{2+}$  uptake by uncoupler [10,29], which was shown to be due to the elimination of the driving force imposed by the opening of the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel. In this case, the compensation of  $\text{Ca}^{2+}$  charge could be accomplished by the efflux of  $\text{K}^+$ . Such an explanation would contradict the established opinion that the RBC membrane does not discriminate between  $\text{Na}^+$  and  $\text{K}^+$  ions [30] and would suggest that the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel is also active under the conditions used in this study. The second explanation is based on the assumption that  $\text{H}^+$  influx is compensated within the pertinent  $\text{Ca}^{2+}$  inward-transporting system. In other words, the transport system conveying  $\text{Ca}^{2+}$  into the cell cytoplasm may be a  $\text{Ca}^{2+}/\text{nH}^+$  antiporter. Such a suggestion may give rise to several questions. First, there is only one protein in the human RBC membrane, which binds the antibody against  $\alpha_1$ -subunit of the  $\text{Ca}^{2+}$  channel has (approximately) the molecular mass corresponding to this channel (i.e.  $\text{Ca}^{2+}$  uniporter) [14]. This fact, however, does not necessarily contradict our results if one takes into account that the slippage phenomenon occurs among ion transport systems (see Ref. [31] for review). Second, the variability of the uncoupler inhibition we observed (Fig. 4B) indicates that other mechanisms of  $\text{H}^+$  gradient compensation are also involved. Such mechanism(s) exist in the RBC membranes. A feasible candidate, the anion channel, which is able to catalyze the  $\text{Cl}^-/\text{OH}^-$  antiport seems to be excluded by the effect of DIDS (Fig. 5) and by the fact that the compensation of the  $\text{H}^+$  influx (due to the activity of the  $\text{Ca}^{2+}$ -ATPase) by the  $\text{Cl}^-/\text{OH}^-$  antiport would cause osmotic effect that could increase the water activity in the cytoplasm and could drive the chloride electrochemical gradient out of equilibrium. Thus, the available data are not sufficient to explain exhaustively the effect of uncoupler on the  $\text{Ca}^{2+}$  influx. However, irrespective of the validity of both explanations of the effect of uncoupler, its effect demonstrates that the influx and efflux of  $\text{Ca}^{2+}$  (in human RBC) are coupled by the RBC  $\text{H}^+$  homeostasis and brings the independent line of evidence that the  $\text{Ca}^{2+}$ -ATPase operates as  $\text{Ca}^{2+}/\text{nH}^+$  antiporter.

## Acknowledgements

This work has been supported by the grant VEGA, no. 2/3188/23 and grant APVT 51-013802.

## References

- [1] E. Carafoli, L. Santella, D. Branca, M. Brini, Generation, control, and processing of cellular calcium signals, *Crit. Rev. Biochem. Mol. Biol.* 36 (2001) 107–260.
- [2] E. Carafoli, Calcium signaling: a tale for all seasons, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 1115–1122.
- [3] M. Brini, E. Carafoli, Calcium signalling: a historical account, recent developments and future perspectives, *Cell. Mol. Life Sci.* 57 (2000) 354–370.
- [4] D.A. Nachshen, M.P. Blaustein, Influx of calcium, strontium, and barium in presynaptic nerve endings, *J. Gen. Physiol.* 79 (1982) 1065–1087.
- [5] P. Massini, E.F. Luscher, On the significance of the influx of calcium ions into stimulated human blood platelets, *Biochim. Biophys. Acta* 436 (1976) 652–663.
- [6] S. Nejedliková, L. Varečka, Characteristics of the passive  $^{45}\text{Ca}^{2+}$  transport by human blood platelets. Their dependence on the monovalent cation composition of the suspension media, *Gen. Physiol. Biophys.* 13 (1994) 29–42.
- [7] V.L. Lew, R.Y. Tsien, C. Miner, R.M. Bookchin, Physiological  $[\text{Ca}^{2+}]_i$  level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator, *Nature* 298 (1982) 478–481.
- [8] M.K. McNamara, J.S. Wiley, Passive permeability of human red blood cells to calcium, *Am. J. Physiol.* 250 (1986) C26–C31.
- [9] N.I. Pokudin, S.N. Orlov, The  $\text{Ca}^{2+}$  transport in human red blood cells: the study with cells loaded with highly selective calcium chelator, *Biol. Membr.* 3 (1986) 108–117 (in Russian).
- [10] L. Varečka, E. Peterajová, J. Ševčík,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel and the activation of  $\text{Ca}^{2+}$  influx in vanadate-treated red blood cells, *Gen. Physiol. Biophys.* 16 (1997) 339–357.
- [11] S.A. Desai, P.H. Schlesinger, D.J. Krogstad, Physiologic rate of carrier-mediated  $\text{Ca}^{2+}$  entry matches active extrusion in human erythrocytes, *J. Gen. Physiol.* 98 (1991) 349–364.
- [12] B. Engelmann, V. Gross, U. Schumacher, J. Duhm, Epidermal growth factor stimulates  $\text{Ca}^{2+}$  uptake of human erythrocytes, *Pflügers Arch.* 421 (1992) 497–502.
- [13] L. Varečka, E. Peterajová, J. Ševčík, Vanadate changes  $\text{Ca}^{2+}$  influx pathway properties in human red blood cells, *Gen. Physiol. Biophys.* 16 (1997) 359–372.
- [14] D.A. Andrews, L. Yang, P.S. Low, Phorbol ester stimulates a protein kinase C-mediated agatoxin TK-sensitive calcium permeability pathway in human red blood cells, *Blood* 100 (2002) 3392–3399.
- [15] L. Soldati, R. Spaventa, G. Vezzoli, S. Zerbi, D. Adamo, A. Caumo, R. Rivera, G. Bianchi, Characterization of voltage-dependent calcium influx in human erythrocytes by fura-2, *Biochem. Biophys. Res. Commun.* 236 (1997) 549–554.
- [16] L. Soldati, D. Adamo, S. Zerbi, A. Caumo, R. Spaventa, G. Bianchi, G. Vezzoli, Erythrocyte voltage-dependent calcium influx is reduced in hemodialyzed patients, *Kidney Int.* 56 (1999) 190–197.
- [17] V. Niggli, E. Sigel, E. Carafoli, The purified Ca pump of human erythrocyte membrane catalyzes an electroneutral  $\text{Ca}/\text{H}$  exchange in reconstituted liposomal systems, *J. Biol. Chem.* 257 (1982) 2350–2356.
- [18] A. Villalobo, B.D. Roufogalis, Proton countertransport by the reconstituted erythrocyte  $\text{Ca}^{2+}$ -translocating ATPase: evidence using ionophoretic compounds, *J. Membr. Biol.* 93 (1986) 249–258.
- [19] M.A. Milanick, Proton fluxes associated with the Ca pump in human red blood cells, *Am. J. Physiol.* 258 (1990) C552–C562.
- [20] B. Gassner, S. Lutterbacher, H.J. Schatzmann, A. Wuthrich, Dependence of the red blood cell calcium pump on the membrane potential, *Cell Calcium* 9 (1988) 95–103.
- [21] P.J. Sims, A.S. Waggoner, C.H. Wang, J.F. Hoffman, Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles, *Biochemistry* 13 (1974) 3315–3330.

- [22] K. Kaiserová, B. Lakatoš, E. Peterajová, J. Orlický, L'. Varečka, Investigation of properties of the  $\text{Ca}^{2+}$  influx and of the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  efflux (Gárdos effect) in vanadate-treated and ATP-depleted human red blood cells, *Gen. Physiol. Biophys.* 21 (2002) 429–442.
- [23] M.C. Wright, R. Issa, D.E. Smart, N. Trim, G.I. Murray, J.N. Primrose, M.J. Arthur, J.P. Iredale, D.A. Mann, Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats, *Gastroenterology* 121 (2001) 685–698.
- [24] P.A. Knauf, G.F. Fuhrmann, S. Rothstein, A. Rothstein, The relationship between anion exchange and net anion flow across the human red blood cell membrane, *J. Gen. Physiol.* 69 (1977) 363–386.
- [25] V.L. Lew, Permeability and permeabilization of red cells to calcium, in: F. Bonner (Ed.), *Intracellular Calcium Regulation*, Alan R. Liss, New York, 1990, pp. 1–17.
- [26] B. Engelmann, J. Duhm, Distinction of two components of passive  $\text{Ca}^{2+}$  transport into human erythrocytes by  $\text{Ca}^{2+}$  entry blockers, *Biochim. Biophys. Acta* 981 (1989) 36–42.
- [27] L'. Varečka, E. Peterajová, E. Pišová, Properties of the  $\text{Ca}^{2+}$  influx reveal the duality of events underlying the activation by vanadate and fluoride of the Gardos effect in human red blood cells, *FEBS Lett.* 433 (1998) 157–160.
- [28] L'. Varečka, E. Peterajová, Activation of red cell  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel by  $\text{Ca}^{2+}$  involves a temperature-dependent step, *FEBS Lett.* 276 (1990) 169–171.
- [29] L'. Varečka, E. Carafoli, Vanadate-induced movements of  $\text{Ca}^{2+}$  and  $\text{K}^+$  in human red blood cells, *J. Biol. Chem.* 257 (1982) 7414–7421.
- [30] V.L. Lew, L. Beaugé, Passive cation fluxes in red cell membranes, in: G. Giebisch, D.C. Tosteson, H.H. Ussing (Eds.), *Membrane Transport in Biology*, Springer, Berlin, 1979, pp. 81–115.
- [31] M.C. Berman, Slippage and uncoupling in P-type cation pumps; implications for energy transduction mechanisms and regulation of metabolism, *Biochim. Biophys. Acta* 1513 (2001) 95–121.